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Direct Detection of *Shigella flexneri* and *Salmonella typhimurium* in Human Feces by Real-Time PCR

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Abstract We have established a SYBR Green-based real-time PCR method using AnyDirect solution, which enhances PCR from whole blood, for direct amplification of the *virA* gene of *Shigella flexneri* and the *invA* gene of *Salmonella typhimurium* from human feces without prior DNA purification. When we compared the efficiency of conventional or real-time PCR amplification of the *virA* and *invA* genes from the supernatant of boiled feces supplemented with *S. flexneri* and *S. typhimurium* in the presence or absence of AnyDirect solution, amplification products were detected only in reactions to which AnyDirect solution had been added. The detection limit of real-time PCR was 1×10^4 CFU/g feces for *S. flexneri* and 2×10^4 CFU/g feces for *S. typhimurium*; this sensitivity level was comparable to other studies. Our real-time PCR assay with AnyDirect solution is simple, rapid, sensitive, and specific, and allows simultaneous detection of *S. flexneri* and *S. typhimurium* directly from fecal samples without prior DNA purification.

Keywords: *Shigella flexneri*, *Salmonella typhimurium*, real-time PCR, human feces

Shigella and *Salmonella* infections are generally diagnosed by isolation and identification of the organisms from fecal cultures. Culture methods are precise, but relatively inefficient, time-consuming, and labor intensive. Polymerase chain reaction (PCR), an alternative approach, may avoid these problems and has proved to be an invaluable method for rapid detection of *Shigella* [4, 7, 8, 15] and *Salmonella* [3, 10, 11, 16, 23]; however, DNA purification is usually unavoidable in order to perform the PCR. Direct PCR without DNA purification is a more rapid and simple approach in which the untreated sample is used directly as a PCR template, thus eliminating the steps of cell recovery and DNA extraction. Direct PCR has been used with bacterial colonies [6, 19, 26], intact cultured bacterial cells [9], yeast colonies [12, 20], and animals [5].

However, direct PCR amplification of DNA from clinical specimens, such as blood and feces, is inefficient and difficult because of the presence of numerous PCR inhibitors including hemin [1], immunoglobulin G [2], anticoagulants [21], bile salts [23], and complex polysaccharides [13]. Purification of template DNA was therefore essential to eliminate PCR inhibitors from clinical samples; and this is a time-consuming and labor-intensive process when a large number of samples need to be analyzed.

Recently, Yang *et al.* [24] developed a novel PCR reaction buffer, called AnyDirect solution (Bioquest, Seoul, Korea), which effectively enhanced PCR from whole blood without prior DNA isolation. In the present study, we evaluated the usefulness of this PCR solution for direct amplification of the *virA* gene of *Shigella flexneri* and the *invA* gene of *Salmonella typhimurium* from human feces by SYBR Green-based real-time PCR without prior DNA purification.

Materials and Methods

Bacterial Strains and Fecal Samples

Shigella flexneri (KCCM 40948) and *Salmonella typhimurium* (KCCM 40253) were obtained from the Korean Culture Center of Microorganisms (KCCM). *S. flexneri* and *S. typhimurium* were grown on blood agar plates at 37°C for 24 h. A portion of one colony was suspended in 0.5 ml of phosphate-buffered saline (PBS), and the number of colony-forming units (CFU) in the suspension was determined by culturing on a blood agar plate for 18 h at 37°C. Suspensions were diluted with PBS and used to spike fecal samples.

Feces used for the sensitivity assay (PCR amplification by spiking) were negative for *S. flexneri* and *S. typhimurium*,

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shown by culture. Informed consent for the use of feces was obtained from participants. Feces were suspended in 10 times volumes (v/w) of PBS and crushed by vortexing. Spiked fecal samples were prepared by mixing the fecal suspension and the appropriately diluted suspension of *S. flexneri* or *S. typhimurium* in PBS. After heating at 100°C for 5 min, the mixture was centrifuged at 8,000 ×*g* for 5 min, and the supernatant was used for PCR.

PCR Primers

Primers used to detect the *virA* gene in *S. flexneri* were developed by Villalobo and Torres [22], and a 215-bp fragment was amplified. Primers used to detect the *invA* gene in *S. typhimurium* were previously published by Rahn *et al.* [17], and a 284-bp fragment was amplified.

Conventional PCR Conditions

Conventional PCR was done in a 20-μl mixture containing serially diluted fecal samples, 1× conventional PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$) or AnyDirect solution (Bioquest, Seoul, Korea), 0.5 μM each of primers, 0.2 mM each of dNTP, and 1 unit of *Taq* DNA polymerase (Bioquest, Seoul, Korea). PCR was performed in a thermal cycler (Clemens, Germany) preheated to 95°C. The cycling conditions were initial denaturation for 7 min at 95°C, followed by 40 cycles of 40 s at 95°C, 40 s at 55°C, 40 s at 72°C, and an additional 10 min at 72°C for final elongation. After the reaction, the PCR mixture (10 μl) was electrophoresed on a 2% agarose gel and stained with ethidium bromide.

Real-Time PCR Conditions

Real-time PCR was performed and analyzed with a Rotor-Gene 6000 system (Corbett Research, Sydney, Australia). The reaction mixtures (20 μl) consisted of serially diluted fecal samples, a 1:75,000 dilution of SYBR Green I (Molecular Probes, Eugene, OR, U.S.A.), 1× conventional PCR buffer or AnyDirect solution (Bioquest, Seoul, Korea), 0.25 μM each of primers, 0.2 mM each of dNTP, and 1 unit of *Taq* DNA polymerase (Bioquest, Seoul, Korea). Amplification involved initial denaturation at 95°C for 5 min followed by 40 cycles of denaturation at 95°C for 30 s, primer annealing at 55°C for 40 s, and extension at 72°C for 30 s. Fluorescence data were acquired at 72°C during each cycle. To determine the specificity of PCR reactions, melt curve analysis was carried out after amplification by slow heating from 72 to 95°C, with fluorescence acquisition at 1°C intervals and a 5-s hold at each increment.

Duplex real-time PCR was performed under the same conditions as above, using fecal samples containing equal amounts of *S. flexneri* and *S. typhimurium* appropriately diluted with PBS.

RESULTS

Conventional PCR Detection of *S. flexneri* from Fecal Samples

To determine whether AnyDirect solution can effectively enhance PCR from feces without DNA purification, we compared the efficiency of conventional PCR amplification of the *Shigella*-specific *virA* gene in the absence and presence of AnyDirect solution using spiked fecal samples. The effect of adding AnyDirect solution to the PCR mix of fecal samples for *S. flexneri* is shown in Fig. 1.

When *S. flexneri* was serially diluted in PBS and each dilution was used as a PCR template in the absence and presence of AnyDirect solution, a 215-bp PCR product was formed in every tube (Fig. 1A). However, when boiled feces (final 1.0% in the PCR mixture) spiked with *S. flexneri* were used as a PCR template, PCR with conventional buffer failed to amplify the *virA* fragment (215 bp) from any samples, indicating that there were residual PCR inhibitors in the boiled feces (Fig. 1B, lanes 1 to 8). In contrast, however, amplification products were detected in reactions to which AnyDirect solution had been added, showing that AnyDirect solution effectively suppressed the residual inhibitors in boiled feces (Fig. 1B, lanes 9 to 16). The minimum number of cells necessary for detection of *S. flexneri* by PCR in fecal samples was 1×10^2 CFU/ml in the presence of AnyDirect solution; therefore, the detection limit is about 1×10^4 CFU/g feces. When the final

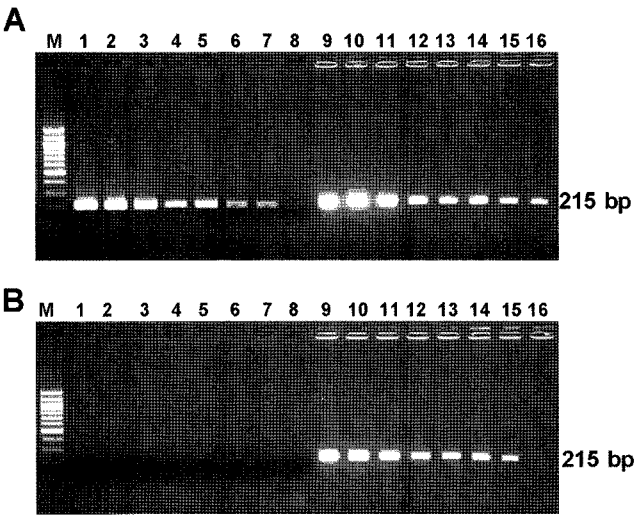

Fig. 1. PCR amplification of *virA* gene fragment (215 bp) with conventional buffer (lanes 1 to 8) or with AnyDirect solution (lanes 9 to 16) from PBS containing *S. flexneri* (**A**) or from feces (1.0% final in the PCR mixture) spiked with *S. flexneri* (**B**): lane M, size marker (100-bp ladder); lanes 1 and 9=6.4×10^6 CFU/ml was present in the PCR mixture; lanes 2 and 10=3.2×10^5 CFU/ml; lanes 3 and 11=1.6×10^4 CFU/ml; lanes 4 and 12=8×10^2 CFU/ml; lanes 5 and 13=4×10^2 CFU/ml; lanes 6 and 14=2×10^2 CFU/ml; lanes 7 and 15=1×10^2 CFU/ml; lanes 8 and 16=5×10^1 CFU/ml.

feces concentration in the PCR mixture was increased to 1.5%, the minimum number of cells required to detect *S. flexneri* in fecal samples was 4×10^2 CFU/ml with a detection limit of 2.7×10^4 CFU/g feces (data not shown). Therefore, a final concentration of 1.0% feces in the PCR mixture produced the best results, and this concentration was used for real-time PCR assays.

Real-Time PCR Detection of *S. flexneri* and *S. typhimurium* from Fecal Samples

The SYBR Green-based real-time PCR assay was then used to detect and quantify *S. flexneri* in fecal samples spiked with various concentrations of target bacteria. When we used spiked fecal samples directly without DNA purification in real-time PCR, amplification products were detected only in reactions to which AnyDirect solution had been added (data not shown). The final fecal concentration in the real-time PCR mixture was 1.0%, and feces did not interfere with fluorescence detection at this concentration.

Fig. 2A shows PCR quantification plots for amplification of the *virA* fragment from spiked fecal samples in the presence of AnyDirect solution. Fig. 2C show the linearity of the standard curves and sensitivity of the real-time PCR assays when serially diluted spiked fecal samples were used as template. Similar to conventional PCR, the detection limit was around 1×10^4 CFU/g feces. Melt curve analysis was carried out in conjunction with each real-time PCR assay to distinguish the fluorescence signal from the specific amplification product from that of nonspecific products. The sharp, single peak in the Fig. 2B indicates that no nonspecific products were formed in the reaction. To verify the melt curve data, the PCR product was assayed by gel electrophoresis, which showed no additional bands as in conventional PCR (data not shown). We performed two additional separate replicate real-time PCR experiments and obtained similar results (data not shown).

To confirm that this SYBR Green-based real-time PCR assay with AnyDirect solution is useful for detection of other bacteria, we applied this assay to the detection and quantification of *S. typhimurium* in fecal samples spiked with various concentrations of target bacteria. Fig. 3 shows PCR quantification plots for the amplification of the *Salmonella*-specific *invA* fragment from fecal samples in the presence of AnyDirect solution (Fig. 3A), the linearity of the standard curves (Fig. 3C) and melt curve analysis (Fig. 3B). The quantification plot and melt curve analysis indicate that the desired PCR product was efficiently amplified in the reaction. To verify the single peak in the melt curve data (T_m=87°C), the PCR product was assayed by gel electrophoresis, which revealed only the desired 284-bp band (data not shown). When the final fecal concentration in the PCR mixture was 1.0%, the minimum number of cells required to detect *S. typhimurium* was 2×10^2 CFU/ml with a detection limit of around 2.0×10^4 CFU/g feces.

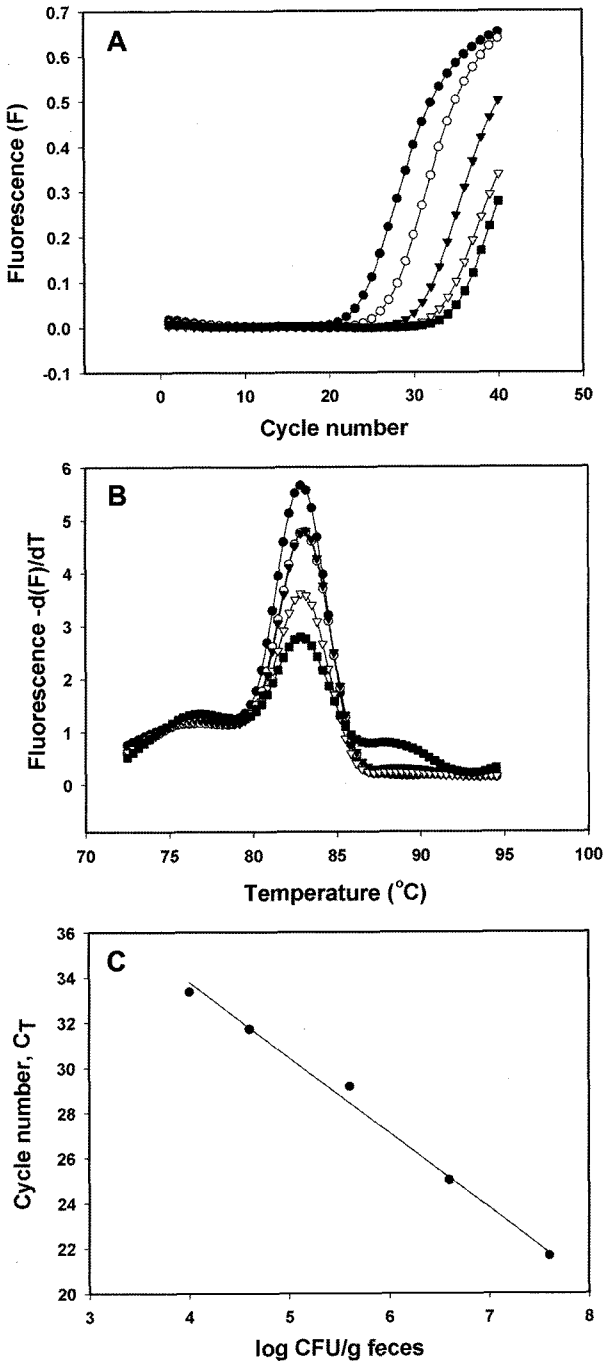

Fig. 2. Real-time PCR amplification of the *virA* gene fragment from fecal samples spiked with *S. flexneri* in the presence of AnyDirect Solution.

(**A**) Amplification profiles and (**B**) Melting curve analysis: 4×10^5 CFU/ml was present in the PCR mixture (●); 4×10^4 CFU/ml (○); 4×10^3 CFU/ml (▼); 4×10^2 CFU/ml (▽); 1×10^2 CFU/ml (■). **C**. Generation of standard curve from the data plotted in panel **A**. The threshold cycles (C_Ts) were plotted against the CFU/g feces. The straight line that is calculated by linear regression shows a square regression coefficient (R^2) of 0.992.

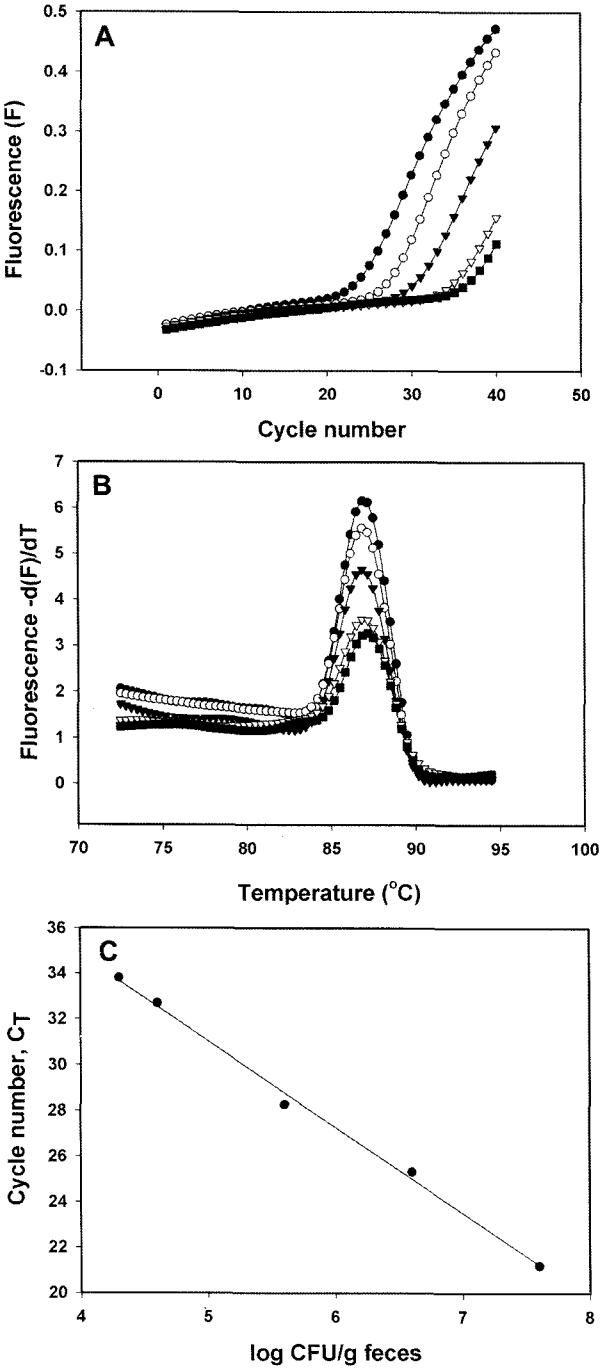

Fig. 3. Real-time PCR amplification of the *invA* gene fragment from fecal samples spiked with *S. typhimurium* in the presence of AnyDirect Solution.

(**A**) Amplification profiles and (**B**) Melting curve analysis: 4×10^5 CFU/ml was present in the 20-μl PCR mixture (●); 4×10^4 CFU/ml (○); 4×10^3 CFU/ml (▼); 4×10^2 CFU/ml (▽); 2×10^2 CFU/ml (■). **C.** Generation of standard curve from the data plotted in panel A (R^2=0.996).

Simultaneous Detection of *S. flexneri* and *S. typhimurium* in Fecal Samples by Duplex Real-Time PCR

Finally, a duplex SYBR Green-based real-time PCR assay was used to simultaneously detect *S. flexneri* and *S. typhimurium* in fecal samples spiked with various concentrations of target bacteria in the presence of AnyDirect solution. PCR products corresponding to *Shigella*-specific *virA* (215 bp, T_m=82°C) and *Salmonella*-specific *invA* (284 bp, T_m= 87°C) were observed, and the curve analysis of the two T_m peaks, corresponding to the two PCR products in the same reaction tube, revealed that they were distinguishable from each other (Fig. 4). Fluorescence from SYBR Green binding to primer-dimers was not a problem, because of the low T_m, which was several degrees (>5°C) below that of the specific products. This demonstrates that target sequences of these two genes from *S. flexneri* and *S. typhimurium* could simultaneously be amplified and detected by our direct duplex real-time PCR assay. The minimum number of cells necessary for simultaneous detection of *S. flexneri* and *S. typhimurium* from fecal samples was 1×10^2 and 2×10^2 CFU/ml, respectively.

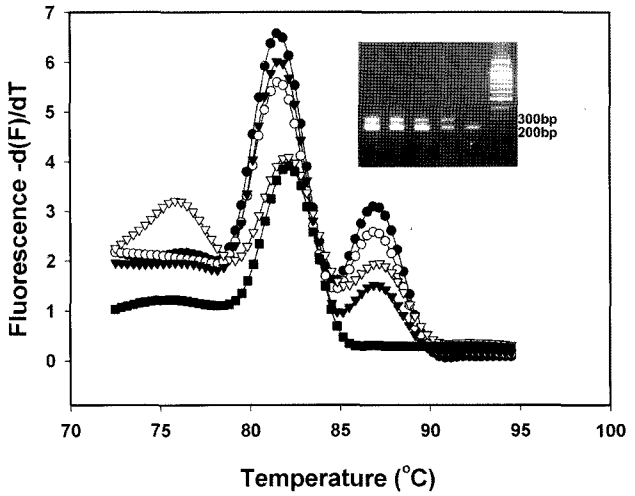

Fig. 4. Melting curve analysis of duplex real-time PCR amplification.

The *virA* and *invA* gene fragments were amplified simultaneously from fecal samples spiked with equal amounts of *S. flexneri* and *S. typhimurium* in the presence of AnyDirect Solution. 4×10^5 CFU/ml was present in the 20-μl PCR mixture (●); 4×10^4 CFU/ml (○); 4×10^3 CFU/ml (▼); 2×10^2 CFU/ml (▽); 1×10^2 CFU/ml (■). T_ms were resolvable between the two products; T_m was 82°C and 87°C for *virA* and *invA*, respectively. Gel analysis of the amplification product in 2% agarose is displayed in the inset. Specific products are visible at 215 bp and 284 bp for each dilution.

DISCUSSION

DNA purification, which is often a pretreatment for PCR, is laborious and impractical for routine procedures. PCR performed directly on clinical samples after a simple pretreatment is preferred because of time saving, convenience, cost, safety for sample handlers to avoid infection, and possible automation for large-scale diagnosis.

Boiling of feces is a very simple pretreatment to release bacterial genomic DNA and to inactivate nucleolytic enzymes,

but is not useful for direct PCR because inhibitors of PCR still remain. However, in the present study, we showed that AnyDirect solution effectively suppressed the residual inhibitors in boiled feces, and conventional and real-time PCR were performed successfully in the presence of AnyDirect solution directly using fecal samples without prior DNA purification.

The sensitivity level achieved in this study was comparable to other studies. Okamoto *et al.* [14] reported direct detection of *E. coli* O157 from heated feces by conventional PCR with an estimated detection limit of about 3×10^4 CFU/g feces. Yavzori *et al.* [25] reported a detection level of 10^4 *Shigella* CFU/g feces, whereas Rychlik *et al.* [18] reported a detection level of 10^5 *Salmonella* CFU/g feces by nested PCR. The detection limit of our real-time PCR was around 1×10^4 CFU/g feces for *Shigella* detection and 2×10^4 CFU/g feces for *Salmonella* detection.

Conventional PCR methods require amplification in a thermocycler and product separation by gel electrophoresis, which increases the time required to obtain results and, more importantly, the risk of contamination. These limitations have led to the development of real-time PCR assays, which avoid the need for post-amplification procedures and reduce the risk of carryover contamination. In addition, real-time PCR can also quantify PCR products with greater reproducibility. However, the efficiency of the real-time PCR is crucial to obtaining reliable quantitative data. In our direct real-time PCR method, successful and efficient real-time PCR required only AnyDirect solution in the PCR mixture.

In conclusion, our direct real-time PCR assay is simple, rapid, sensitive, and specific, and allows simultaneous detection of *Shigella* and *Salmonella* directly from fecal samples without prior DNA purification. The rapidity of our real-time PCR method also makes it convenient to be used in clinical laboratories for diagnosing *Shigella* and *Salmonella* infections, and the method could be applicable to detection of other bacteria in fecal samples as well.

Acknowledgments

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References

1. Akane, A., K. Matsubara, H. Nakamura, S. Takahashi, and K. Kimura. 1994. Identification of the heme compound copurified with deoxyribonucleic acid (DNA) from bloodstains, a major inhibitor of polymerase chain reaction (PCR) amplification. *J. Forensic Sci.* **39:** 362–372.
2. Al-Soud, W. A., L. J. Jonsson, and P. Radstrom. 2000. Identification and characterization of immunoglobulin G in blood as a major inhibitor of diagnostic PCR. *J. Clin. Microbiol.* **38:** 345–350.
3. Chiu, C. H. and J. T. Ou. 1996. Rapid identification of *Salmonella* serovars in feces by specific detection of virulence genes, *invA* and *spvC*, by an enrichment broth culture-multiplex PCR combination assay. *J. Clin. Microbiol.* **34:** 2619–2622.
4. Frankel, G., L. Riley, J. A. Giron, J. Valmassoi, A. Friedmann, N. Strockbine, S. Falkow, and G. K. Schoolnik. 1990. Detection of *Shigella* in feces using DNA amplification. *J. Infect. Dis.* **161:** 1252–1256.
5. Grevelding, C. G., A. Kampkotter, M. Hollmann, U. Schafer, and W. Kunz. 1996. Direct PCR on fruitflies and blood flukes without prior DNA isolation. *Nucleic Acids Res.* **24:** 4100–4101.
6. Gussow, D. and T. Clackson. 1989. Direct clone characterization from plaques and colonies by the polymerase chain reaction. *Nucleic Acids Res.* **17:** 4000.
7. Islam, D. and A. A. Lindberg. 1992. Detection of *Shigella dysenteriae* type 1 and *Shigella flexneri* in feces by immunomagnetic isolation and polymerase chain reaction. *J. Clin. Microbiol.* **30:** 2801–2806.
8. Islam, M. S., M. S. Hossain, M. K. Hasan, M. M. Rahman, G. Fuchs, D. Mahalanabis, A. H. Baqui, and M. J. Albert. 1998. Detection of *Shigellae* from stools of dysentery patients by culture and polymerase chain reaction techniques. *J. Diarrhoeal Dis. Res.* **16:** 248–251.
9. Joshi, A. K., V. Baichwal, and G. F. Ames. 1991. Rapid polymerase chain reaction amplification using intact bacterial cells. *Biotechniques* **10:** 42–45.
10. Jung, S. J., H. Kim, and H. Kim. 2005. Quantitative detection of *Salmonella typhimurium* contamination in milk, using real-time PCR. *J. Microbiol. Biotechnol.* **15:** 1353–1358.
11. Lim, S., K. Yong, and S. Ryu. 2005. Analysis of *Salmonella* pathogenicity island 1 expression in response to the changes of osmolarity. *J. Microbiol. Biotechnol.* **15:** 175–182.
12. Ling, M., F. Merante, and B. H. Robinson. 1995. A rapid and reliable DNA preparation method for screening a large number of yeast clones by polymerase chain reaction. *Nucleic Acids Res.* **23:** 4924–4925.
13. Monteiro, L., D. Bonnemaison, A. Vekris, K. G. Petry, J. Bonnet, R. Vidal, J. Cabrita, and F. Megraud. 1997. Complex polysaccharides as PCR inhibitors in feces: *Helicobacter pylori* model. *J. Clin. Microbiol.* **35:** 995–998.
14. Okamoto, H., E. Takano, T. Sugao, K. Kage, E. Okamoto, N. Nishimura, and K. Ueda. 1999. Direct amplification of *Escherichia coli* O157 vero toxin genes from human faeces by the polymerase chain reaction. *Ann. Clin. Biochem.* **36:** 642–648.
15. Park, S., H. Kim, and H. Kim. 2006. Simultaneous detection of *Yersinia enterocolitica*, *Staphylococcus aureus*, and *Shigella* spp. in lettuce using multiplex PCR method. *J. Microbiol. Biotechnol.* **16:** 1301–1305.

16. Park, S., K. Seo, and S. Ha. 2007. A response surface model based on absorbance data for the growth rates of *Salmonella enterica* serovar typhimurium as a function of temperature, NaCl, and pH. *J. Microbiol. Biotechnol.* **17:** 644–649.
17. Rahn, K., S. A. De Grandis, R. C. Clarke, S. A. McEwen, J. E. Galan, C. Ginocchio, R. Curtiss 3rd, and C. L. Gyles. 1992. Amplification of an *invA* gene sequence of *Salmonella typhimurium* by polymerase chain reaction as a specific method of detection of *Salmonella*. *Mol. Cell. Probes* **6:** 271–279.
18. Rychlik, I., L. van Kesteren, L. Cardova, A. Svestkova, R. Martinkova, and F. Sisak. 1999. Rapid detection of *Salmonella* in field samples by nested polymerase chain reaction. *Lett. Appl. Microbiol.* **29:** 269–272.
19. Sandhu, G. S., J. W. Precup, and B. C. Kline. 1989. Rapid one-step characterization of recombinant vectors by direct analysis of transformed *Escherichia coli* colonies. *Biotechniques* **7:** 689–690.
20. Sathe, G. M., S. O'Brien, M. M. McLaughlin, F. Watson, and G. P. Livi. 1991. Use of polymerase chain reaction for rapid detection of gene insertions in whole yeast cells. *Nucleic Acids Res.* **19:** 4775.
21. Satsangi, J., D. P. Jewell, K. Welsh, M. Bunce, and J. I. Bell. 1994. Effect of heparin on polymerase chain reaction. *Lancet* **343:** 1509–1510.
22. Villalobo, E. and A. Torres. 1998. PCR for detection of *Shigella* spp. in mayonnaise. *Appl. Environ. Microbiol.* **64:** 1242–1245.
23. Widjojoatmodjo, M. N., A. C. Fluit, R. Torensma, G. P. Verdonk, and J. Verhoef. 1992. The magnetic immuno polymerase chain reaction assay for direct detection of *Salmonellae* in fecal samples. *J. Clin. Microbiol.* **30:** 3195–3199.
24. Yang, Y., J. Y. Kim, Y. Song, and D. Kim. 2007. A novel buffer system, AnyDirect, can improve polymerase chain reaction from whole blood without DNA isolation. *Clin. Chim. Acta* **380:** 112–117.
25. Yavzori, M., D. Cohen, R. Wasserlauf, R. Ambar, G. Rechavi, and S. Ashkenazi. 1994. Identification of *Shigella* species in stool specimens by DNA amplification of different loci of the *Shigella* virulence plasmid. *Eur. J. Clin. Microbiol. Infect. Dis.* **13:** 232–237.
26. Zon, L. I., D. M. Dorfman, and S. H. Orkin. 1989. The polymerase chain reaction colony miniprep. *Biotechniques* **7:** 696–698.